REMARKS/ARGUMENTS

Claims 6-13 are active.

Support for the claims is found in original Claims 1-5, pages 2-4 and 7-10 of the specification as originally filed.

No new matter is believed to have been added by the submission.

The objection to claim 5 is no longer applicable.

The rejection to claims 1-5 under 35 USC 112, second paragraph is no longer applicable. Further, there is nothing indefinite about the phrasing used in the present claims: "A is a C_{1-8} alkylene or a C_{1-8} alkylene substituted with a hydroxyl group."

The rejection of Claim 5 under 35 USC 112, first paragraph is no longer applicable. The claims presented herein define treating neutrophilia or COPD with the term "preventing" deleted.

The rejection of Claims 1-5 under 35 USC 102(b) in view of the Egi patent (US '758) is no longer applicable as those claims have been cancelled. Further, Egi teaches angiogenesis activities but neither describes or suggests treating neutrophilia or COPD as is claimed.

Withdrawal of the rejection is requested.

The rejection of Claims 1-5 under 35 USC 102(b) in view of the Tanikawa patent (US '883) is no longer applicable as those claims have been cancelled. Further, Tanikawa teaches

anti-thrombotic, cadiotonic, vasodilation, and anti-SRS-A activities but neither describes or suggests treating neutrophilia or COPD as is claimed.

For the same reasons, the claims of Tanikawa do not render the present claims obvious as cited in the obviousness-type double patenting rejection.

Withdrawal of the rejections is requested.

The rejection of Claims 1-5 under 35 USC 103(a) citing the Doherty patent (US '346) is no longer applicable as Claims 1-5 have been cancelled. While Doherty does discuss phosphodiesterase (PDE) inhibitors, the treatment of neutrophilia or COPD as is claimed would not have been obvious because as is explained below different type PDE's have vastly different effects with little, if any, expectation as to how one inhibitor for a particular type of PDE will respond to another type PDE.

Doherty et al disclose a compound recited in Claim 1 that is a type V phosphodiesterase (PDE) inhibitor and in the cited col. 3, Doherty describes that PDE inhibitors are effective in treating a variety of disorders, including obstructive lung disease.

Doherty et al primarily is concerned with inhibitors of type V PDEs, among various PDE subtypes (note Doherty's discussion of the subtypes in col. 3, lines 11-20). Doherty's inhibitors are particularly effective in treating erectile dysfunction.

Although Doherty et al refer "Goodman and Gilman's The Pharmacological Basis of Therapeutic Ninth Edition, Chapter 34" in column 3, lines 6-7, for a variety of therapeutic uses of PDE inhibitors, Chapter 34 of the Goodman merely relates to relation between PDEs and heart diseases and doesn't provide any substantive information for the treatment of neutrophilia or COPD with 3(2H)-pyridazinone compound represented by the formula (I) as is claimed.

When the present application was filed, one of the claimed compounds had already been reported to inhibit PDE3 and PDE5, and its IC50 values against PDE3 and PDE5 (0.04 μm and 0.07 μM, respectively) reported in Ikegawa et al ($(P_3$-112), *The Japanese Journal of Pharmacology* vol. 67, Supplement 1(1995) (copy attached)) indicate that the compound inhibits mainly PDE3 and but also PDE5. Further, a detailed report on their selectivity shows that the PDE3 inhibitory activity of the compound accounts for most of its PDE inhibitory activity (see Ishiwata et al, *Life Sciences* 81 (2007) 970-978 (copy attached)).

In contrast, it is PDE4 inhibitors that has been expected to be effective in treatment of COPD at the time the present application was filed. See "Within the airways, PDE4 appears to be the most important isoenzymes because of its distribution in airway smooth muscle and inflammatory cells" on page 100, the right column, lines 25-27 in *Clinical and Experimental Allergy*, 1999 Volume 29, Supplement 2, pages 99-109 (copy attached).

Therefore, the claimed 3(2H)-pyridazinone compound represented by the formula (I) would not have been expected to be effective in treatment of COPD because it is not a PDE4 inhibitor, as discussed above.

When the present application was filed, no definite conclusion has been made on whether PDE5 inhibition is effective in treatment of COPD. For example, *Clinical and Experimental Allergy* at pages 104 left column, the 4th to 10th lines from the bottom describes that "while clinical trials which were performed with the PDE5 selective inhibitor zaprinast showed that oral administration of the compound reduced exercise-induced bronchoconstrictiori in adult asthmatics. Surprisingly, in the same studies the drug had no effect on histamine-induced bronchoconstriction in the same subjects, nor did it affects exercise-induced bronchoconstriction in children." Thus, it could not have been common knowledge in this technical field that PDE5 inhibitors are effective in treatment of COPD.

As far as the applicants are aware, no prior art references suggest therapeutic effect of PDE3 inhibitors on COPD. Indeed, *Clinical and Experimental Allergy*, page 104, the left column, lines 36-37 describes that selective PDE3 inhibitors such as milrinone) "appeared unlikely to be benefit in the therapy of obstructive lung diseases" on page 104, the left column, lines 36-37.

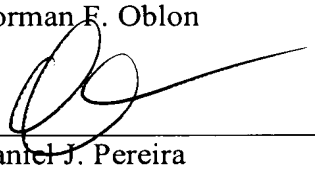
Therefore, while there is a suggestion that PDE inhibitors of general type MAY be used to treat chronic obstructive lung disease, there was no *per se* information that lead one to expect that the claimed page 104, the left column, lines 36-37 would be effective in treating neutrophilia or COPD given the peculiarities of the different classes of PDEs, PDE inhibitors and that the art shows a pattern that inhibition of one class for certain disorders provides no reasonable expectation of how that inhibitor will effect other PDE types or disorders correlating with that type.

Withdrawal of the rejection is requested.

A Notice of Allowance is also requested.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.
Norman F. Oblon



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P3-109 Ameliorative effects of vasodilative prostaglandins against thrombus-induced circulatory disorder in rat femoral artery.

Katsumi Yamanaga, Toshiaki Akira, Takeshi Uchida, Masahiro Watanabe and Yoshio Kagitani. Central Research Laboratory, Green Cross Corporation, Osaka 573, Japan.

This study was designed to evaluate the effects of PGE1 and PGI2 on arterial thrombus-induced reduction in the blood flow in rat hind paw. Severe or moderate ischemia was respectively applied to rat hind paw by thrombus-formation in the common or superficial femoral artery, which were initiated by endothelial insult through the combined irradiation of Xe light with rose bengal injection. After thrombus formation, the regional blood flow in hind paw decreased and lipo-PGE1 or carbacyclin (stable PGI2 derivative) was intravenously injected at the doses of 0.3-10 μg/kg. In the severe model, both drugs had no effect on critically decreased regional blood flow in thrombus-occluded side, although in unoccluded side, they significantly increased the blood flow. In the moderate model, cumulative application of lipo-PGE1 significantly restored the decreased blood flow to normal in occluded side in dose dependent manner. Carbacyclin inclined to improve the decreased blood flow with less potency than that of lipo-PGE1, although there was no significant difference in thrombus-formation in between the arteries applied both drugs. Thus, lipo-PGE1 may be expected to ameliorate insufficient peripheral circulation more effectively than the PGI2-analog.

P3-110 ONO-1301, a novel non-prostanoid prostacyclin mimetic with a potent inhibitory activity against thromboxane synthase.

Shin-ichi Yamazaki, Akihiro Kawamoto, Michihiko Hayashi, Masakazu Ohnemia, Kei Machii, Masao Naka, Kimio Kondo and Nobuyuki Hamanaka. Bioscience Division of Discovery Research Laboratories-I, Minase Research Institute, Ono Pharmaceutical Co., Ltd., Shimamoto, Mishima, Osaka 618, Japan.

We have recently developed a novel non-prostanoid prostacyclin mimetic, (7,8-dihydro-5-[(E)-2-[(α-(3-pyridyl)benzylidene) aminooxy]ethyl]-1-naphthyl-oxy] acetic acid (ONO-1301) possessing a potent inhibitory activity against thromboxane (TX) synthase. ONO-1301 inhibited the binding of [3H]-iloprost for human platelet membranes (IC50=0.18 μM) and increased cyclic AMP level in human platelets. ONO-1301 also inhibited human platelet TX synthase activity (IC50=0.026 μM). The platelet aggregations *in vitro* with platelet rich plasma (PRP) obtained from human, monkey, dog, rat and rabbit were inhibited by ONO-1301 (IC50=0.181-27.7 μM). To compare the inhibitory activities of this compound on platelet aggregation and TX synthase, we performed *ex vivo* study to examine 1) ADP-induced platelet aggregation with rat PRP and 2) arachidonic acid-induced TX production in rat whole blood. Oral administration of ONO-1301 inhibited platelet aggregation and TX synthase activity in a dose-dependent manner, and both inhibitory effects of ONO-1301 were coincidentally observed at the dose of more than 10 mg/kg. Furthermore, oral administration of ONO-1301 (≥30mg/kg) reduced systemic blood pressure in conscious rats. Intra-duodenal administration of ONO-1301 (≥10mg/kg) increased the skin blood flow of hind limb in anesthetized rats. Oral administration of ONO-1301 (10mg/kg, twice a day) suppressed the progression of tissue necrosis in the laurate-induced peripheral circulatory disease in rats. These findings suggest, that ONO-1301 is a novel prostacyclin mimetic with the inhibitory activity against TX synthase and may be a useful agent for a therapy of peripheral circulatory insufficiencies and other circulatory diseases in clinical stage.

P3-111 Effect of prostacyclin on splenic diameter and blood pooling in dogs.

Katsuhiko Noguchi, Yoshihiko Ojiri*, Toshihiro Matsuzaki, Junko Nakasone, Miyoko Uza** and Mamo Sakanashi. Department of Pharmacology, School of Medicine and *Laboratories of Physiology & Pharmacology and **Community Health Nursing, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, Okinawa 903-01, Japan.

Effect of intra-arterial (i.a.) administration of prostacyclin (PGI_2) on splenic diameter (SpD) and arterial and venous blood cell counts was evaluated in anesthetized dogs. The main splenic artery and vein were dissected for measurement of splenic arterial blood flow (SpF) and i.a. administration, and for sampling of splenic venous blood, respectively. SpD was continuously measured by sonomicrometry. Counts of white blood cell (WBC), red blood cell (RBC) and platelet (Plt) in blood sampling from the aorta and splenic vein were estimated by an automatic blood cell counter. Bolus injections of PGI_2 (1-100 ng/kg, i.a.) produced dose-dependent increases in SpF and SpD associated with significant decreases in splenic venous concentrations of WBC, RBC and Plt. Similar changes in SpD and blood cell counts were also observed under a constant SpF. Infusion of PGI_2 (100 ng/kg/min, i.a.) caused a marked increase in SpD with immediate reductions in venous concentrations of WBC, RBC and Plt followed by significant reductions in those in arterial blood. These results indicate that PGI_2 produces potent and flow-independent splenic dilation that may contribute to a decrease in circulating blood cell concentration.

P3-112 Pharmacological profiles of NM-702, a novel multiple inhibitor of cyclic-nucleotide phosphodiesterases and thromboxane A_2 synthase

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NM-702, 4-Bromo-5-(3-pyridylmethylamino)-6-[3-(4-chlorophenyl)propoxy]-3(2H)pyridazinone, is a novel antiplatelet agent. In this study, pharmacological profiles of NM-702 were evaluated in vitro and were compared with those of cilostazol, a cyclic-nucleotide phosphodiesterase (PDE) type III(cAMP-inhibited PDE) specific inhibitor. The inhibitory effects of NM-702 against human platelet aggregation induced by various agonists were 200-500 times stronger than those of cilostazol. NM-702 inhibited not only PDE type III but also type V(cGMP-specific PDE) which are both richly distributed in platelets, with the IC_{50} values of 0.04 and 0.07 μM, respectively. In human platelets, NM-702 increased both cAMP- and cGMP-levels in a concentration-dependent manner (10^{-9}-10^{-6} M), whereas, cilostazol did not increase the cGMP-level. Interestingly, NM-702 caused a potent inhibition of thromboxane A_2 (TXA_2) synthase (IC_{50}; 6.7 nM). In conclusion, the multiple inhibitory action of above enzymes might contribute to the potent antiplatelet action of NM-702.

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NT-702 (parogrelil hydrochloride, NM-702), a novel and potent phosphodiesterase inhibitor, improves reduced walking distance and lowered hindlimb plantar surface temperature in a rat experimental intermittent claudication model

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Abstract

NT-702 (parogrelil hydrochloride, NM-702), 4-bromo-6-[3-(4-chlorophenyl)propoxy]-5-[(pyridin-3-ylmethyl)amino]pyridazin-3(2H)-one hydrochloride, a novel phosphodiesterase (PDE) inhibitor synthesized as a potent vasodilatory and antiplatelet agent, is being developed for the treatment of intermittent claudication (IC) in patients with peripheral arterial disease. We assessed the efficacy of NT-702 in an experimental IC model as compared with cilostazol and additionally investigated the pharmacological property *in vitro* and *ex vivo*. NT-702 selectively inhibited PDE3 (IC_{50}=0.179 and 0.260 nM for PDE3A and 3B) more potently than cilostazol (IC_{50}=231 and 237 nM for PDE3A and 3B) among recombinant human PDE1 to PDE6. NT-702 inhibited *in vitro* human platelet aggregation induced by various agonists (IC_{50}=11 to 67 nM) and phenylephrine-induced rat aortic contraction (IC_{50}=24 nM). Corresponding results for cilostazol were 4.1 to 17 μM and 1.0 μM, respectively. NT-702 (3 mg/kg or more) significantly inhibited *ex vivo* rat platelet aggregation after a single oral dose. For cilostazol, 300 mg/kg was effective. In a rat femoral artery ligation model, NT-702 at 5 and 10 mg/kg repeated oral doses twice a day (BID) for 13 days significantly improved the reduced walking distance while the lowered plantar surface temperature was improved at 2.5 mg/kg and more. Cilostazol also improved the walking distance and surface temperature at 300 mg/kg BID but significant difference was only observed for surface temperature on day 8. These results suggest that NT-702 can be expected to have therapeutic advantage for IC.

Keywords: Phosphodiesterase inhibitor; Recombinant human protein; Vasodilation; Platelet aggregation; Rats; Intermittent claudication

Introduction

Peripheral arterial disease (PAD) is a common manifestation of systemic atherosclerosis. Patients with PAD should be considered to have an increased risk of cardiovascular events such as myocardial infarction, ischemic stroke and vascular death (CAPRIE Steering Committee, 1996; Ness and Aronow, 1999; Hiatt, 2001; Norgren et al., 2007). Intermittent claudication (IC), accompanied by reproducible ischemic leg pain, is one of the most common symptoms of PAD, which severely restricts walking activity and lowers quality-of-life (QOL). Medical treatment including antiplatelet drugs and antihyperlipidemic drugs is effective to reduce the subsequent cardiovascular risk but does not affect symptoms of IC (Hiatt, 2001, 2002; Norgren et al., 2007).

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There are currently two phosphodiesterase (PDE) inhibitors, pentoxifylline and cilostazol, approved for the treatment of IC in several countries including the US and the UK. While therapeutic efficacy of pentoxifylline is limited, cilostazol has been shown to improve walking ability and QOL in patients with IC significantly (Dawson et al., 1998, 2000; Money et al., 1998; Beebe et al., 1999; Norgren et al., 2007) but more effective therapeutic drugs are still required.

NT-702 (parogrelil hydrochloride, NM-702), 4-bromo-6-[3-(4-chlorophenyl)propoxy]-5-[(pyridin-3-ylmethyl)amino]pyridazin-3(2H)-one hydrochloride was synthesized as a potent vasodilatory and antiplatelet agent. Although the potency and selectivity of NT-702 against human PDEs remains unknown, the result from basic pharmacological study suggests that the compound selectively inhibits PDE3 and 5 purified from rabbit heart and platelets (Ikegawa et al., 1995). Recently, the result of phase II/III clinical trial of NT-702 for the treatment of IC in patients with PAD was disclosed. NT-702 significantly improved patients' walking activity as compared with placebo and was well tolerated (Brass et al., 2006). For this reason, it is of great interest whether NT-702 has therapeutic advantage over other drugs approved for IC.

In this study, we assessed the efficacy of NT-702 in a rat femoral artery ligation model of IC as compared with cilostazol as well as *in vitro* and *ex vivo* pharmacological properties including inhibitory activity against human recombinant PDEs.

Materials and methods

All studies reported here were reviewed by the Ethics Review Committee for Animal Experimentation of Nissan Chemical Industries and Taisho Pharmaceutical Animal Care Committee and meet the Japanese Experimental Animal Research Association Standards as defined in the Guidelines for Animal Experiments (1987).

Drugs and chemicals

NT-702 (4-bromo-6-[3-(4-chlorophenyl)propoxy]-5-[(pyridin-3-ylmethyl)amino]pyridazin-3(2H)-one hydrochloride, Fig. 1) was synthesized at Nissan Chemical Industries (Tokyo, Japan). Cilostazol was extracted and purified from Pletaal® tablets (Otsuka Pharmaceutical Co., Tokyo, Japan) or purchased from Huangyan Botai Chemical (Huangyan, China) or Sigma-Aldrich (St. Louis, MO, USA). Adenosine 3′, 5′-cyclic monophosphate (cAMP) sodium salt monohydrate, guanosine 3′, 5′-cyclic monophosphate (cGMP) sodium salt and bovine thrombin were purchased from Sigma-Aldrich. [5′, 8-^{3}H]cAMP ([^{3}H]cAMP) ammonium salt and [8-^{3}H]cGMP ([^{3}H]cGMP) ammonium salt were purchased from

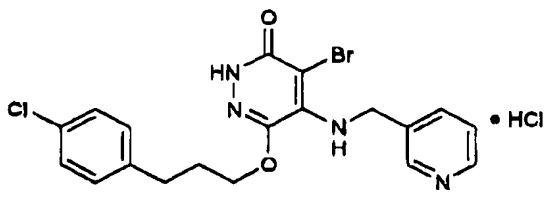

Fig. 1. Chemical structure of NT-702.

GE healthcare Bio-Sciences (Little Chalfont, UK). Collagen (collagen reagent Horm) was purchased from Nycomed (Munich, Germany). U46619 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Platelet activating factor (PAF) was purchased from Biomol International (San Diego, CA, USA). L-phenylephrine hydrochloride and papaverine hydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were the highest grade commercially available.

PDE inhibition assay

Recombinant human (rh) PDE enzymes (PDE1A3, PDE2A3, PDE3A, PDE3B, PDE4B2, PDE4D1, PDE5A1 and PDE6αβ) expressed in a baculovirus/Sf9 expression system were purchased from Scottish Biomedical Ltd. (Glasgow, UK). The PDE activity was determined radiometrically by using PDE [^{3}H]cAMP SPA Enzyme Assay kit or PDE [^{3}H]cGMP SPA Enzyme Assay kit (GE healthcare Bio-Sciences) on 96-well Isoplate (PerkinElmer, Wellesley, MA, USA) as described previously (Wang et al., 1997). Briefly, after preincubation of the assay mixture contained various concentrations of NT-702 or cilostazol dissolved in DMSO and the enzyme solution for 5 min at room temperature, the reaction was started by the addition of substrate solution (cAMP or cGMP) at a final concentration of Km value for each enzyme. Sufficient amount of enzyme to achieve 10% substrate breakdown was added. After incubation for an additional 30 min at 30 °C, the reaction was terminated by the addition of yttrium silicate SPA beads suspension. The plates were counted for radioactivity by MicroBeta Trilux (PerkinElmer). The concentration causing 50% inhibition (IC_{50}) of PDEs was calculated. In our preliminary study, 3-isobutyl-1-methylxanthine (IBMX) for PDE1, erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride salt (EHNA) for PDE2, cilostazol for PDE3, rolipram for PDE4 and sildenafil for PDE5 and PDE6 used as positive control showed inhibitory activities against each enzyme with IC_{30} values similar to those previously reported (data not shown, Podzuweit et al., 1995; Loughney et al., 1996; Bolger et al., 1997; Huston et al., 1997; Ballard et al., 1998; Sudo et al., 2000; Zoraghi et al., 2006).

In vitro human platelet aggregation assay

Human blood samplings for platelet aggregation study were performed after obtaining written informed consent. Blood was collected from healthy human volunteers by venipuncture and instantly mixed with 1/10 volume of 3.8% trisodium citrate and centrifuged at 160 ×*g* for 10 min to prepare platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained from the precipitated fraction of PRP by centrifugation at 2000 ×*g* for 15 min. PRP was washed with Hepes–Tyrode's buffer (137 mM NaCl, 2.6 mM KCl, 1.0 mM $MgCl_2$, 12.1 mM $NaHCO_3$ and 37.8 mM HEPES, pH 6.5) containing 7.7 mM EDTA by centrifugation at 600 ×*g* for 10 min. The platelet pellet was resuspended in Hepes–Tyrode's buffer (pH7.35) containing 11 mM glucose, 1 mM $CaCl_2$ and 3.5 g/L BSA to prepare washed platelet (WP). PRP was used for assessing the ADP, collagen, U46619 and PAF-induced platelet aggregation, and WP was for thrombin.

Platelet aggregation was measured by the method of Born (Born, 1962) using an aggregometer (Hematracer 601, Niko Bioscience, Tokyo, Japan). A sample of PRP or WP was incubated with NT-702 or cilostazol dissolved in DMSO for 2 min at 37 °C and then each agonist was added. The concentration of each agonist was adjusted to a minimal concentration to induce maximal aggregation. The light transmission on the aggregation response curve was measured for 7 min. The parameters of the extent of platelet aggregation were taken as the area under the time-aggregation curve in case of collagen and the maximum aggregation on the aggregation curve in case of other agonists. The IC_{50} values of platelet aggregation were calculated from concentration-inhibition curve.

In vitro vasorelaxation assay in rat aorta

Rat thoracic aortae were isolated by procedures previously described (Noguchi et al., 1998). Male Wistar rats (Charles River Japan, Inc., Yokohama, Japan) weighing 214 to 240 g were used. All rats were sacrificed by venesection under pentobarbital sodium anesthesia. The aortae were excised and placed in well-oxygenated (95% O_2–5% CO_2), modified Krebs–Henseleit solution (KHS) (118.0 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25.0 mM $NaHCO_3$ and 11.1 mM glucose, pH 7.4). To avoid the interference of endothelium-derived factors, intimal surface of the ring preparations were gently rubbed using a cotton swab moistened with KHS.

The ring preparations were then mounted using stainless hooks under the optimal resting tension of 1 g in a 10-ml organ bath containing well-oxygenated KHS at 37 °C. The tension changes of the ring preparation were isometrically recorded with a force-displacement transducer (TB-612T, Nihon Kohden, Tokyo, Japan) connected to a polygraph (WT-685G, Nihon Kohden). After the ring preparations were contracted with phenylephrine (0.3 μM), NT-702 or cilostazol dissolved and diluted in DMSO were added cumulatively to the bath medium. Papaverine (100 μM) was added to the bath to determine the maximum vasorelaxation, after treatment of NT-702 or cilostazol at the highest concentration. The values of IC_{50} for NT-702 and cilostazol were determined from each preparation.

Ex vivo rat platelet aggregation assay

Male Wistar rats (8 weeks old, Charles River Japan, Inc.) were used in this study. NT-702 or cilostazol was suspended in 0.5% methylcellulose (MC) and orally administered to rats fasted overnight. Blood was drawn from the abdominal aorta under pentobarbital sodium anesthesia with a plastic syringe containing 3.8% trisodium citrate (1/10 volume) at 1, 3 and 6 h after the administration. Each blood sample was centrifuged at 270 ×*g* for 7 min to obtain PRP. Platelet counts were adjusted to 5×10^5 cells/μL using autologous PPP. After the PRP was preincubated at 37 °C for 2 min in an aggregometer, platelet aggregation was assessed by adding ADP (10 μM) or collagen (10 μg/mL) over a 7-min period. Platelet aggregation was expressed as area under the time-aggregation curve.

Rat femoral artery ligation model

Male Wistar rats (Keari, Osaka, Japan) weighing 259 to 371 g were subject to treadmill walking test using a custom-made apparatus (NeuroScience, Tokyo, Japan). Walking speed, started at 15 m/min, was increased by 5 m/min every 5 min. Walking distance was defined as the total distance until the animal fell off the apparatus three times. Furthermore, the animals were anesthetized with diethyl ether, and the plantar surface temperatures of the hindlimb were measured by a thermography apparatus (TVS-8000, Nihon Avionics, Tokyo, Japan). The hindlimb site for the temperature measurement was the middle point of the line connecting the roots of the first and fifth fingers. Plantar surface temperature was expressed as the difference in temperature between the right and left hindlimbs. Treadmil and thermography measurements were performed on 2 to 4 days before the ligation. Animals satisfying both of two conditions including walking distance (150 m or more but 400 m or less) and plantar surface temperature (within 1 °C) were used as the objects for construction of a model of femoral artery ligation.

After the animals were selected, the rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). The right femoral artery was exposed and ligated with silk suture. A second ligation was applied at about 1 cm peripherally of the first ligation. Following the ligation at two places, animals were sutured after applying a penicillin G (Mycillin sol, Meijiseika, Tokyo, Japan) to the incision.

One day after ligation (Day 1), the walking distance was evaluated with the same method mentioned above. The animals for which the walking distance was shortened to 50% of the distance before ligation were assigned to experimental groups and also subjected to the measurement of hindlimb plantar

Table 1
Inhibitory effects of NT-702 and cilostazol on recombinant human PDEs

IC_{50}(nM)									
	PDEs	PDE1A3	PDE2A3	PDE3A	PDE3B	PDE4B2	PDE4D1	PDE5A1	PDE6αβ
	substrate	cGMP	cAMP	cAMP	cAMP	cAMP	cAMP	cGMP	cGMP
NT-702		3340 (2220–5030)	137 (108–173)	0.179 (0.0568–0.562)	0.260 (0.0780–0.870)	1240 (799–1920)	1620 (1430–1840)	87.2 (67.9–112)	129 (88.4–189)
cilostazol		>50,000	32,200 (28,800–36,000)	231 (59.6–895)	237 (16.7–3360)	>50,000	>50,000	9080 (7690–10,700)	>50,000

IC_{50} values and 95% C.I. were calculated according to the non-linear regression analysis. Data are expressed as the mean of 4 separate experiments. The values in parentheses indicate 95% C.I.

surface temperature by the method mentioned above. From Day 2, NT-702 (2.5, 5 and 10 mg/kg) or cilostazol (300 mg/kg) were orally administered twice a day (BID) for 13 days (until Day 14). 0.5% MC was used as the vehicle. Treadmill and thermography measurements were performed on Day 4, Day 8 and Day 15 in addition to on Day -2 ~ -4 (before ligation) and Day 1.

Statistical analysis

The results are expressed as mean±S.E.M. or alternatively 95% confidence interval (C.I.). Statistical analyses were performed by non-linear regression analysis for the calculation of 95% C.I., Dunnett's test for the *ex vivo* study and for the study of NT-702 in the rat femoral artery ligation model and unpaired t-test for the study of cilostazol in the model between vehicle-treated and test drug-treated groups. All of statistical analyses were performed by using SAS system ver.8.2 (SAS institute Japan, Tokyo, Japan). A p value less than 0.05 was considered statistically significant.

Results

PDE inhibitory activity

We measured the inhibitory effect of NT-702 and cilostazol against rhPDE1 to PDE6. NT-702 strongly inhibited PDE3A and 3B, and the IC_{50} values (0.179 and 0.260 nM, respectively) were much smaller than those for other enzymes (Table 1). Cilostazol also inhibited PDE3A and 3B selectively (231 and 237 nM for PDE3A and 3B, respectively), but the inhibitory effect of NT-702 was about 1000 times more potent than that of cilostazol (Table 1).

Inhibitory effects on human platelet aggregation in vitro

The inhibitory effects of NT-702 and cilostazol on human platelet aggregation were examined. NT-702 potently and concentration-dependently inhibited human platelet aggregation induced by ADP (5–20 μM), collagen (1–2 μg/mL), U46619 (0.5–1 μM), PAF (0.5–2 μM) and thrombin (0.1 units/mL) with IC_{50} values (nM) of 67, 11, 12, 21 and 31, respectively (Table 2). Although cilostazol also suppressed platelet aggregation induced by these agonists, the potency of cilostazol was less than that of NT-702. The inhibitory effect of NT-702 was 250 to 520 times more potent than that of cilostazol.

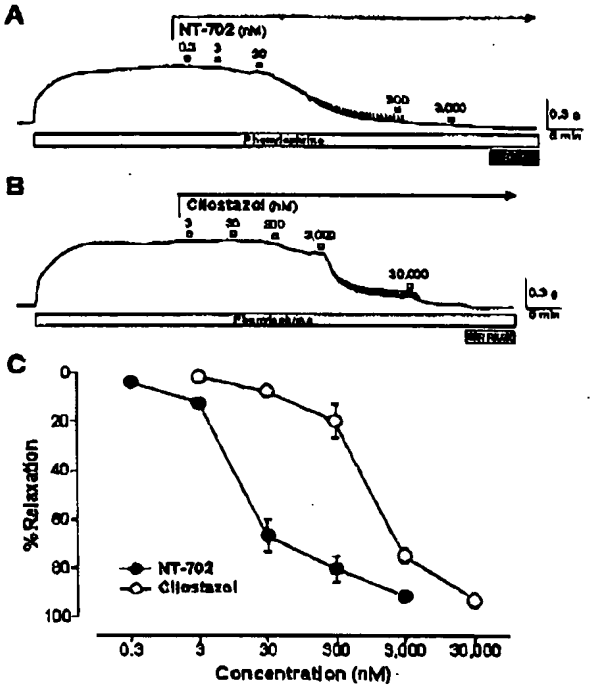

Fig. 2. Vasorelaxation effects of NT-702 and cilostazol on rat aortic rings. A, B; Typical traces showing the relaxant effects of NT-702 and cilostazol on isolated rat aortae without endothelium. After the phenylephrine (0.3 μM)-induced contraction (open column), NT-702 or cilostazol were added cumulatively to the bath medium. Papaverine at 100 μM (PPV; closed column) was added to the bath after treatment of NT-702 or cilostazol at the highest concentration. C; Concentration-response curve for relaxant effects of NT-702 (solid circles, $n=4$) and cilostazol (open circles, $n=8$) on phenylephrine-induced contraction of isolated rat aortae. The vasorelaxant effect of each compound was expressed as a percentage of the maximum relaxation induced by PPV. Data are expressed as the mean±S.E.M.

Vasorelaxation effects on rat aorta

The vasorelaxation effects of NT-702 and cilostazol on rat aortic rings without endothelium were examined. Typical traces showing the relaxant effects of NT-702 (Fig. 2A) and cilostazol (Fig. 2B) were represented. NT-702 inhibited the 0.3 μM phenylephrine-induced contraction in a concentration-dependent manner (Fig. 2A and C). Cilostazol also inhibited the contraction (Fig. 2B and C). The mean IC_{50} values of NT-702

Table 2
Inhibitory effects of NT-702 and cilostazol on human platelet aggregation induced by various aggregating agents

IC_{50}(nM)	ADP	Collagen	U46619	PAF	Thrombin
NT-702	67	11	12	21	31
	(46–98)	(5.7–21)	(5.3–28)	(11–41)	(22–43)
cilostazol	17,000	5700	4100	6800	12,000
	(8900–33,000)	(3700–8800)	(1300–13,000)	(3300–14,000)	(7300–19,000)

IC_{50} values and 95% C.I. were calculated according to the non-linear regression analysis. Data are expressed as the mean of 3–5 human volunteers. Values in parentheses indicate 95% C.I.

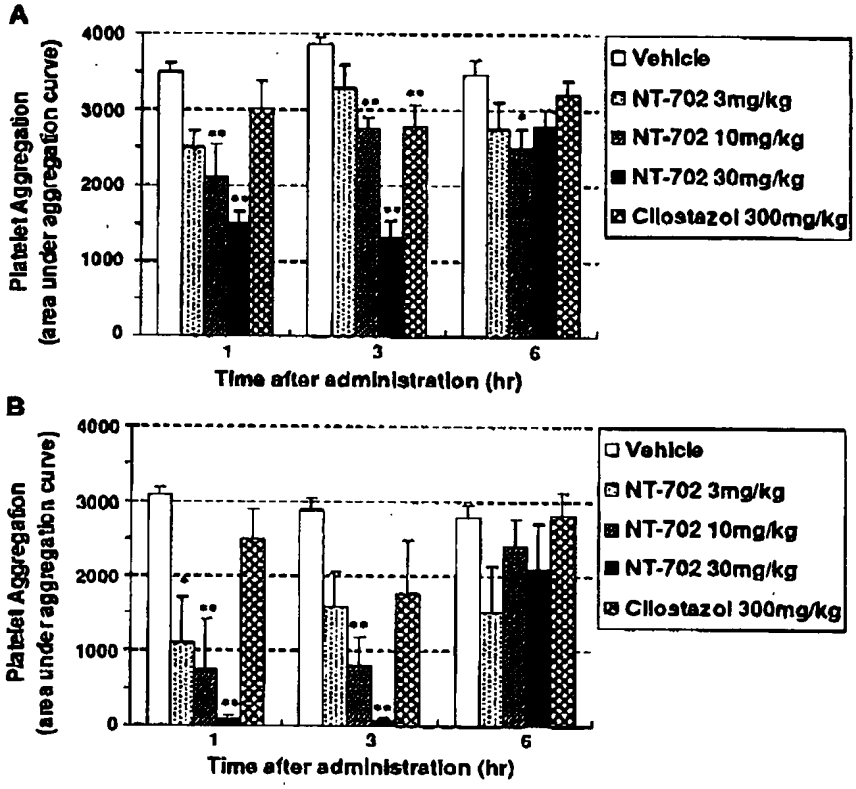

Fig. 3. Effects of orally administered NT-702 and cilostazol on ADP (A) and collagen (B)-induced *ex vivo* platelet aggregation in rat. Citrated blood samples were collected 1, 3 and 6 h after oral administration of NT-702 or cilostazol. Platelet aggregation was assessed by adding 10 μM ADP or 10 μg/mL collagen over a 7-min period and recorded as the area under the time-aggregation curve. A; ADP-induced platelet aggregation. B; collagen-induced platelet aggregation. Data are expressed as the mean±S.E.M. of 5 animals. Statistical analyses were performed using Dunnett's test. $^{*}P<0.05$, $^{**}P<0.01$ vs. control (vehicle group).

and cilostazol were 24 nM (95% C.I.:12–51 nM, $n=4$) and 1000 nM (95% C.I.:570–1900 nM, $n=8$), respectively, suggesting that the vasorelaxant activity of NT-702 was 42 times more potent than that of cilostazol.

Ex vivo inhibitory effects on rat platelet aggregation

The time course of the anti-platelet aggregation effect of NT-702 at doses of 3 to 30 mg/kg after a single oral administration was examined in comparison with cilostazol at a dose of 300 mg/kg. NT-702 at 10 and 30 mg/kg significantly inhibited ADP-induced platelet aggregation, and the inhibitory effect continued for 6 and 3 h, respectively (Fig. 3A). NT-702 significantly inhibited collagen-induced platelet aggregation at 3 to 30 mg/kg and, at 10 and 30 mg/kg, the inhibitory effect continued for 3 h (Fig. 3B). Cilostazol suppressed ADP-induced platelet aggregation at 3 h after the dose of 300 mg/kg (Fig. 3A).

Effects on rat femoral artery ligation model

We examined the efficacy of NT-702 and cilostazol in a rat femoral artery ligation model when the drugs were orally administered BID for 13 days. In the treadmill walking test, the walking distance on an average in each group before femoral artery ligation was between 242 m at minimum and 282 m at maximum. There was no difference among the groups. After ligation, the reduction of walking distance between 151 m at minimum and 184 m at maximum was recognized on Day 1 in each group. In the vehicle-treated group, the walking distance showed the tendency to recover gradually but did not reach to the pre-ligation level until Day 15 (Fig. 4A and B). NT-702 at 5 and 10 mg/kg significantly improved the reduced walking distance on Day 8 (Fig. 4A) as compared with vehicle-treated group. Although cilostazol at 300 mg/kg, in contrast, showed an improving tendency, significant differences were not observed on any day (Fig. 4B).

In the thermography test, there was no difference in the surface temperature of the both hindlimb plantae before ligation (data not shown). The temperature in ligated hindlimb planta decreased by 10–12 °C after ligation, and in the vehicle-treated group it hardly recovered until Day 15 (Fig. 5A and B). NT-702 at 2.5 mg/kg significantly improved the lowered plantar surface temperature on Day 8 (Fig. 5A) as compared with vehicle-treatd group. Moreover, NT-702 at 5 and 10 mg/kg significantly improved the lowered surface temperature on Day 8 and Day 15. On the other hand, cilostazol at 300 mg/kg showed a

significant improvement only on Day 8 (Fig. 5B). Representative examples of thermogram are shown in Fig. 6.

These results indicated that NT-702 but not cilostazol significantly prolonged the walking distance with improvement of the lowered plantar surface temperature in a rat model of femoral artery ligation.

Discussion

It was recently reported that NT-702 significantly improved patients' walking activity as compared with placebo (Brass et al., 2006). Therefore, it is of great interest whether NT-702 has therapeutic advantage over other drugs approved for IC. In this study, we evaluated the efficacy of NT-702 and cilostazol in a rat femoral artery ligation model to assess the possibility of NT-702 having clinical advantages in IC. In this model, NT-702 significantly and strongly improved the reduced walking distance as well as the lowered plantar surface temperature at doses of 2.5 to

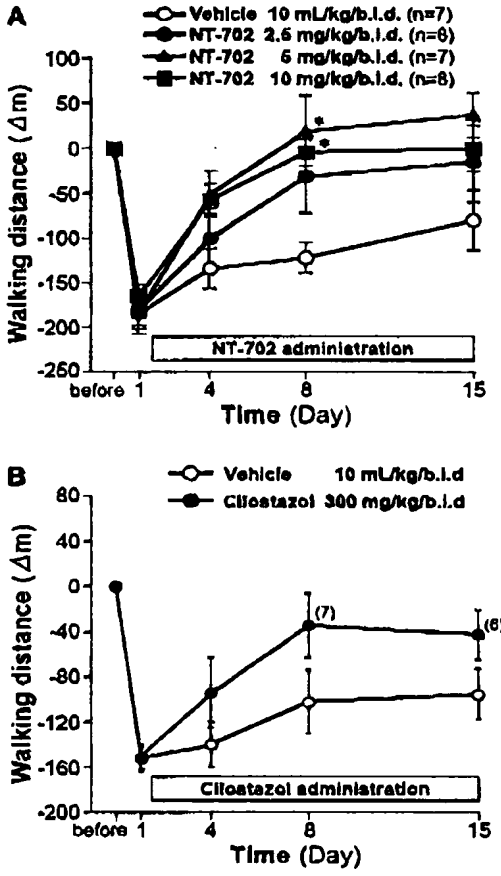

Fig. 4. Effects of NT-702 (A) and cilostazol (B) on walking distance in a rat model of femoral artery ligation. Data are expressed as the mean±S.E.M. of 7–8 rats (A) and 8 rats (B). Numbers in parentheses indicate the number of animals at the specified time points. $^{*}p<0.05$ significantly different from vehicle-treated group (Dunnett's test). There was no significant difference between vehicle- and cilostazol-administered groups (unpaired *t*-test).

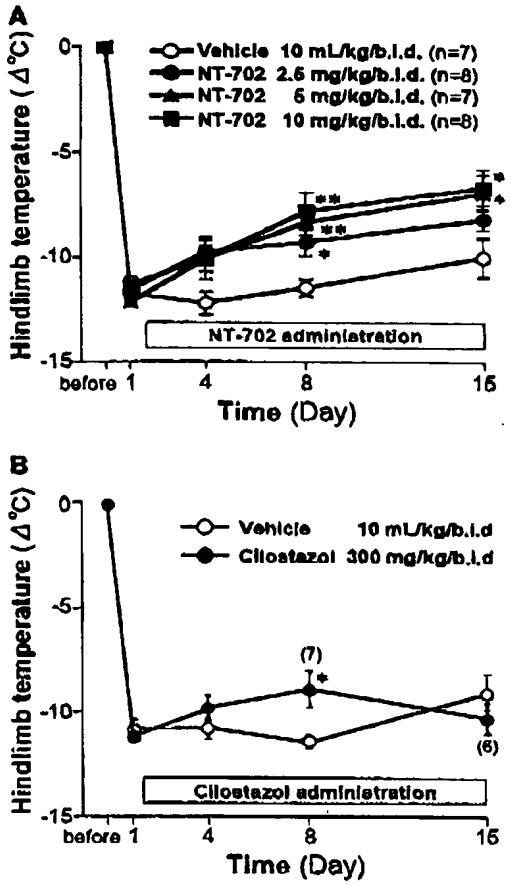

Fig. 5. Effects of NT-702 (A) and cilostazol (B) on surface temperature at hindlimb plants in a rat model of femoral artery ligation. Data are expressed as the mean±S.E.M. of 7–8 rats (A) and 8 rats (B). Numbers in parentheses indicate the number of animals at the indicated time points. $^{*}p<0.05$, $^{**}p<0.01$ significantly different from vehicle-treated group (Dunnett's test). $^{*}p<0.05$ significantly different from vehicle-treated group (unpaired *t* test).

10 mg/kg BID for 13 days as compared with vehicle-treated group. On the other hand, cilostazol, a selective PDE3 inhibitor, approved for the treatment of IC in the US and the UK showed a mild efficacy in our model at a dose of 300 mg/kg BID for 13 days and improvement of the lowered plantar surface temperature was only statistically significant at day 8. The efficacy of NT-702 and cilostazol was not compared directly because in this study the two experiments shown in Fig. 4 were performed independently. However, we believe that the results have a specific meaning as they were obtained using the exact same animal model. To estimate from the result of a rat *ex vivo* experiment, antiplatelet activity of NT-702 at a dose of 2.5 mg/kg is comparable to that of cilostazol at a dose of 300 mg/kg which is almost the higher limit in terms of commonly used dose for *in vivo* experiments of cilostazol. We did not statistically compare the *ex vivo* efficacy of NT-702 directly to one of cilostazol due to these two drugs having different pharmacokinetic properties, particularly the time to maximum plasma concentration when they were orally administered.

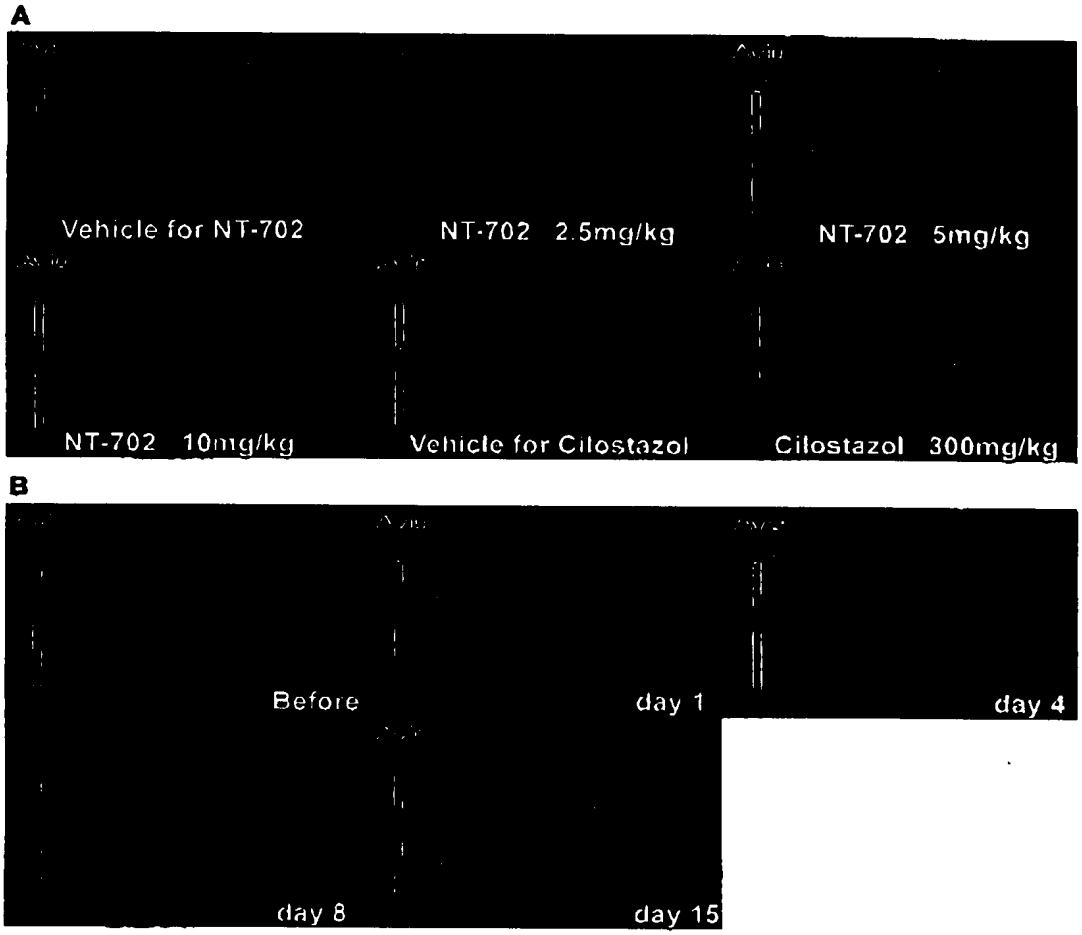

Fig. 6. Representative examples of thermogram on day15 (A) and time course of NT-702 10 mg/kg group (B) after hindlimb ligation. (A) upper left; vehicle for NT-702 study, upper middle; NT-702 2.5 mg/kg, upper right; NT-702 5 mg/kg, bottom left; NT-702 10 mg/kg, bottom middle; vehicle for cilostazol study and bottom right; cilostazol 300 mg/kg. (B) upper left; before ligation, upper middle; day 1, upper right; day 4, bottom left; day 8 and bottom middle; day 15 of NT-702 10 mg/kg.

Orito et al. (2004) has shown the efficacy of cilostazol for IC using a rat femoral artery ligation model which resembles the one being reported here. In their model, cilostazol improved the reduced walking performance at doses of 30 and 100 mg/kg BID. Their model is established by single ligature of the femoral artery, while in our model two ligatures cause a more severe blood flow disturbance. Recently, beraprost sodium has been shown efficacy in improving the walking activity in a rat bilateral ligation model (Miyamoto et al., 2006). Moreover, in the vehicle control group, the reduced walking performance was recovered to almost normal range within 8 days after the ligation. The time course and magnitude of the improvement of walking performance in the vehicle control group of this bilateral and single ligature model is quite similar to the results of our model. Herzog et al. (Herzog et al., 2002) reported that vascular proliferation was notable around the ligation site 24 h after the femoral artery ligation in rats. The extent of the proliferation and the number of collateral vessels were significantly increased 7 days after the ligation. These results suggest that the reduced walking performance (time or distance) in the ligated rats without any drug or vehicle could be partially improved in a time-dependent manner and through the sufficiently developed collateral arterial network around the ligation site. Additionally, it is reported that walking performance is gradually and time-dependently improved in the vehicle control as well as in the drug treatment group in patients with IC (Brass et al., 2006). Although the severity of the ligation models varies, the fact that cilostazol showed a tendency to improve the reduced walking performance suggests that the model in this study is suitable for the evaluation of drug candidates for the treatment of IC.

PDEs play a major role in cell signaling by hydrolyzing cAMP and cGMP. There have been identified 11 different PDE families which constitute the PDE superfamily (Lugnier, 2006). In an experiment using rhPDE1 to PDE6, we found that NT-702 selectively and potently inhibited PDE3 (IC_{50}=0.179 and 0.260 nM for PDE3A and 3B). The selectivity of NT-702 to PDEs was similar to that of cilostazol (Sudo et al., 2000). PDE3A, which hydrolyzes cAMP, is mainly distributed in

platelets, vascular smooth muscle, heart and oocytes whereas PDE3B is mainly in adipocytes, hepatocytes and spermatocytes (Lugnier, 2006). The fact that NT-702 potently inhibited human platelet aggregation induced by multiple agonists and phenylephrine-induced vasocontraction of rat aorta could be explained by the potent PDE3A inhibitory activity of the compound. Our previous basic pharmacological study suggested that NT-702 had a possibility to inhibit PDE5 at the same potency as PDE3 from the experiment using enzymes purified from rabbit tissues (Ikegawa et al., 1995). The discrepancy in the inhibitory activity against PDE3 between the two studies may be due to the species differences or purity of the enzymes. We consider that the result of this study using recombinant human enzymes would reflect the phenomena in human subjects more properly. Moreover, NT-702 inhibited platelet aggregation 250 to 520 times more potently and showed a vasorelaxant activity 42 times more potent than cilostazol. It has been reported that PDE3A from human and rat were quite similar in structure (Degerman et al., 1997) as well as in tissue expression and localization including cardiovascular system (Liu and Maurice, 1998). Therefore, functional contributions of PDE3 inhibitors to human could be comparable with those to rat. Indeed, in our preliminary study, NT-702 and cilostazol inhibited rat platelet aggregation *in vitro* with the same potency as in the case of human, respectively.

These results indicate that NT-702 improved peripheral circulation of ischemic hindlimb and the reduced walking performance through the potent antiplatelet and vasorelaxant activities which can be explained by at least PDE3A inhibition. In addition, the potent activity of NT-702 in this IC model makes us think of possible contribution of other mechanisms. For example, it has been reported that NT-702 inhibited thromboxane synthetase in human platelets and consequently reduced the production of thromboxane A_2 (Ikegawa et al., 1995) which strongly induces platelet aggregation and vasocontraction (Moncada and Vane, 1978). Further studies are needed to fully understand the mechanism of NT-702 in IC and other diseases.

Conclusion

Our results show that NT-702 has potent vasodilatory and antiplatelet activity and one of these mechanisms of actions is considered to be PDE3A inhibition. NT-702 improved the reduced walking distance and the lowered hindlimb plantar surface temperature in a rat experimental IC model while the efficacy of cilostazol was less potent. These results suggest that NT-702 can be expected to have therapeutic advantage for IC.

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Selective phosphodiesterase inhibitors for the treatment of bronchial asthma and chronic obstructive pulmonary disease

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Summary

Theophylline is commonly used in the treatment of obstructive airway diseases. The identification and functional characterization of different phosphodiesterase (PDE) isoenzymes has led to the development of various isoenzyme-selective inhibitors as potential anti-asthma drugs. Considering the distribution of isoenzymes in target tissues, with high activity of PDE3 and PDE4 in airway smooth muscle and inflammatory cells, selective inhibitors of these isoenzymes may add to the therapy of chronic airflow obstruction. However, initial data from clinical trials with selective PDE3 and PDE4 inhibitors have been somewhat disappointing and have tempered the expectations considerably since these drugs had limited efficacy and their use was clinically limited through side effects. The improved understanding of the molecular biology of PDEs enabled the synthesis of novel drugs with an improved risk/benefit ratio. These 'second generation' selective drugs have produced more promising clinical results not only for the treatment of bronchial asthma but also for the treatment of chronic obstructive pulmonary disease.

Keywords: phosphodiesterase inhibitors, phosphodiesterase isoenzymes, theophylline, inflammation asthma, chronic obstructive pulmonary disease

Background

Nonselective inhibitors of cyclic nucleotide phosphodiesterase (PDE), such as theophylline, have been used for the treatment of obstructive airways diseases for several decades. Until now, theophylline remains one of the world's most widely used medications for asthma and plays an important role in the treatment of chronic obstructive pulmonary disease (COPD) [1], despite its relegation from first-line therapy in international guidelines for the treatment of asthma [2].

The renewed interest in recent years regarding the pharmacology and clinical effects of this class of drugs is based largely on advances in the knowledge and understanding of the PDE isoenzymes, which represent one of theophylline's biochemical targets, and from demonstrations of a range of effects of theophylline in addition to its well characterized bronchodilator property. These additional actions are thought to involve a variety of actions such as the effects on the vasculature in patients with increased pulmonary artery pressure, the suppression of inflammatory cell responses, and the induction of apoptosis of eosinophils.

The immunopharmacology of theophylline has become a particular subject of interest in recent years, and the ability of drugs that inhibit certain PDE isoenzymes (see below) to suppress both immune cell functions *in vitro* and allergic pulmonary inflammation *in vivo*, is believed to present an opportunity for the development of novel drugs with anti-inflammatory properties through greater selectivity and improved side-effect profile, compared with nonselective drugs such as theophylline.

Although it has been suggested that some of theophylline's adverse effects are due to the drug's non PDE-inhibitory actions, such as antagonism of adenosine receptors, there is now evidence to suggest that the most common side-effects, including cardiac dysrhythmias and nausea, are also mediated by PDE inhibition.

With this in mind, it was proposed that selective targetting of the PDE isoenzymes involved in regulating cyclic nucleotide levels in cells relevant to asthma and COPD –

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namely bronchial smooth muscle and the inflammatory cells characteristic of bronchial infiltrates – would allow the beneficial effects of theophylline to be obtained in the absence of cardiovascular, gastrointestinal, and central nervous system side-effects. Many such family-selective or 'second-generation' PDE inhibitors have been developed, some exhibiting selectivity ratios of several thousand for a particular isoenzyme family over all other families.

While it has been widely suggested that increasingly selective PDE4 inhibitors will enable targeted suppression of inflammation, several pharmaceutical companies have followed this course in developing selective drugs for clinical trials in asthma and inflammatory diseases [3]. However, in the past clinical trials with some of the highly selective drugs were disappointing. One reason for this limited success may be the presence of significant amounts of other PDE isoenzymes in addition to PDE4 in the target tissues [4,5]. Therefore, just as combined inhibition of PDE3 and PDE4 is more effective than inhibition of a single isoenzyme family in relaxing pre-contracted human airways smooth muscle [6,7], a more effective suppression of inflammation might also be achieved by the combination of selective PDE inhibitors rather than by a single isoenzyme-selective PDE inhibitor. This report tries to summarize current concepts and available clinical data for selective PDE inhibitors for the treatment of asthma and COPD.

Molecular biology of PDE

Cyclic nucleotide PDE was identified 40 years ago as an ubiquitous enzyme catalysing the breakdown of the intracellular 'second messenger' adenosine 3′:5′-cyclic monophosphate (cAMP) and guanosine 3′:5′-cyclic monophosphate (cGMP). Theophylline was recognized as an inhibitor of this enzyme activity and has been shown to exert many of its important therapeutic effects through PDE inhibition. In recent years, PDE has been subject to intense biochemical scrutiny, and is now acknowledged to represent the activity of a large 'superfamily' of enzymes forming at least eight distinct families of isoenzymes that differ in their substrate specificities and affinities and in their sensitivity to stimulation or inhibition by endogenous cofactors and modulators. Theophylline (1,3-dimethylxanthine) inhibits these isoenzymes with roughly equal potency and, in common with several related alkylxanthine compounds such as IBMX (3-isobutyl-1-methylxanthine) and pentoxifylline (1–5-oxohexyl-3–7-dimethylxanthine), is described as a nonselective or 'first generation' PDE inhibitor.

During the last decades, long after the discovery of cyclic AMP and its degrading enzymes, a lot of research concentrated on further characterization of different PDE isoenzymes [8], their localization, and their involvement in the regulation of different cell functions. In the period from 1970 to 1980, various PDE isoenzymes were defined by differences in substrate specificities and/or regulation characteristics. Subsequently, an extensive research and development effort led to the synthesis, characterization, and clinical study of a series of new compounds that were selective for one or two isoenzymes. These mono- and dual-selective 'second-generation' PDE inhibitors were isoenzyme-targetted, especially to PDE3 and PDE4, and they offered both new clinical uses and the possibility for analyses of the tissue distribution and functional role of individual isoenzymes. It emerged that these compounds exhibited a limited organ specificity and – importantly, from a clinical viewpoint – a therapeutic potential against heart failure and depression. Currently, a variety of new substances are under investigation as alternative potential anti-inflammatory drugs and, if the theoretical concept holds true, agents with lower potential for side-effects are being discovered.

In the meantime, eight PDE isoenzyme gene families have been identified and the list is still likely not to be complete [4,9–13]. They not only differ in their substrate specificity, in their affinity, and in their sensitivity to stimulation or inhibition by endogenous cofactors and modulators, but also in their localization in specific organ systems or tissues [12]. Within the airways, PDE4 appears to be the most important isoenzyme because of its distribution in airway smooth muscle and inflammatory cells (Figure 1).

PDE4 has been shown to include the products of four separate genes (PDE4A–D), with variants of PDE4A, 4B, and 4D also occurring through differential mRNA splicing [11]. The expression of the different gene products is heterogeneous and it is striking that PDE4C is rarely expressed in blood-derived cells, including the inflammatory cells implicated in allergic airways obstruction [3,11]. Most of the selective inhibitors developed so far are substrate-site-directed competitive inhibitors, but a few act at allosteric sites [14,15]. Drug targetting can focus either on the selectivity for 'subtypes' of the PDE gene families, e.g. PDE4A, 4B, 4C, or 4D [16], or on one of two conformers of the isoenzyme [17–19], since studies support the proposal that two distinct and catalytically active conformers of PDE4 isoenzymes coexist. One of them binds rolipram at the catalytic site with a high affinity ('high-affinity rolipram binding site') and a second binds rolipram with a lower affinity ('low-affinity rolipram binding site') [18,20]. It is noteworthy that the therapeutic effects appear to be related to the 'low-affinity rolipram binding site', whereas the side-effects are more likely to be related to the 'high-affinity rolipram binding site'. Second-generation PDE4 inhibitors such as RP73401, CDP840, and SB207499 were specifically designed to have an improved therapeutic index according to the approach described above.

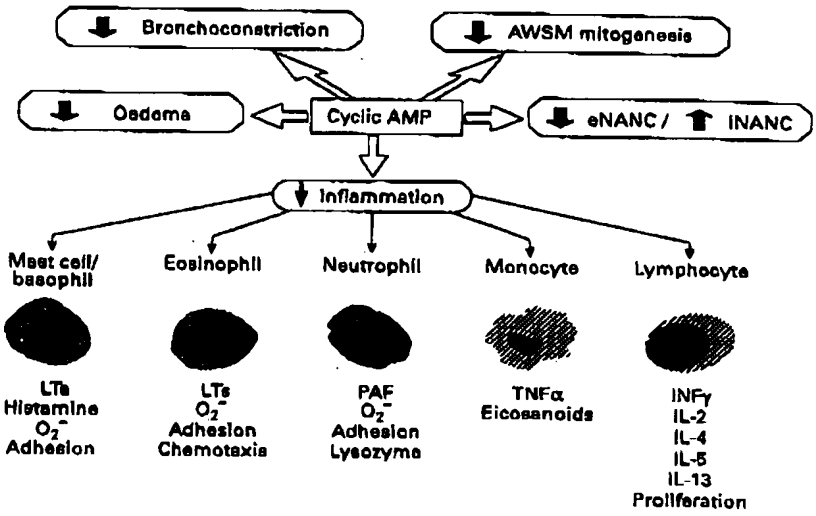

Fig. 1. Effects of cAMP elevation by type 4 selective PDE inhibitors with relevance to the treatment of bronchial asthma and COPD [12]. ↓, inhibition; ↑, stimulation; AWSM, airway smooth muscle; eNANC, excitatory nonadrenergic noncholinergic neurotransmission; iNANC, inhibitory nonadrenergic noncholinergic neurotransmission; LTs, leukotrienes; O_2^-, superoxide anion; PAF, platelet-activating factor; TNFα, tumour necrosis factor alpha; INF-γ, interferon-gamma; IL, interleukin.

Subtype-selective PDE4 inhibitors have yet to be reported. Although the presence of large amounts of PDE4C in the central nervous system, compared with its absence in leucocytes [21,22], suggests that theoretically one promising way forward is to synthesize compounds with decreased activity against this subtype, it is unclear whether inhibiting a single PDE4 subtype will have a great enough impact on total cellular cAMP metabolism to alter cell function meaningfully, and, additionally, inhibition of one subtype might lead to a reciprocal up-regulation of another.

Cellular distribution of PDE isoenzymes

Most studies to date have focused on PDE4 as a potential target for novel anti-inflammatory drugs, since this isoenzyme family is represented in all cells, with the exception of platelets, that are thought to be involved in inflammatory reactions [23]. PDE4, the low Km cAMP-specific isozyme, is the major cAMP-metabolizing enzyme in immune and inflammatory cells, and is one of two major cAMP-metabolizing enzymes in airway smooth muscle. Several inflammatory cell classes have undergone similar investigation, revealing the exclusive presence of PDE4 in neutrophils, eosinophils, basophils, and monocytes, while in macrophages PDE3 and PDE1 and in T lymphocytes PDE7 activity has also been demonstrated [24–26].

Furthermore, PDE inhibitors can, for example, inhibit mediator release of inflammatory cells like monocytes and monocyte-derived macrophages [27], lung mast cells [28,29], T lymphocytes [30,31], B lymphocytes [32], alveolar macrophages [33–35], and eosinophils [3,36,37].

Smooth muscle

Within human airways, isoenzyme-selective PDE inhibitors were identified that cause effective relaxation of airway smooth muscle [6,7,38–40]. Studies of the PDE isoenzyme profiles of these tissues and cells revealed the presence of enzyme activities of the PDE1, PDE2, PDE3, PDE4, and PDE5 classes [6,7,39], while selective inhibitors of PDE3 and PDE4 have been shown to cause relaxation of bronchial rings under various conditions [7,38,41]. Functional analyses of PDE isozymes suggest that PDE3 and PDE4 coregulate cAMP content in human airway smooth muscle. This appears to be the case, because either a combination of PDE3 and PDE4 inhibitors or dual PDE3/4 inhibitors produce a much greater bronchorelaxant effect than individual isoenzyme-selective agents alone [6,39].

Furthermore, a number of studies support the proposal that cAMP not only is involved in smooth muscle relaxation but also exerts an overall inhibitory influence on airway smooth muscle proliferation [42–45]. It can modulate airway smooth muscle hypertrophy and hyperplasia, which are common morphological features of chronic asthma [46,47]. The actions of isoenzyme-selective inhibitors on human airway smooth muscle proliferation have not been investigated; however, in pig aortic smooth muscle the combination of a PDE3 and PDE4 inhibitor, but not the individual inhibitor alone, was shown to have a marked inhibitory effect on proliferation [48].

Four PDE isoenzyme families, PDE1, PDE3, PDE4, and PDE5, have been found in human pulmonary arteries with high quantities of PDE3 and PDE5 and a smaller amount of PDE4 [49]. Selective inhibitors of PDE3 and PDE5, or

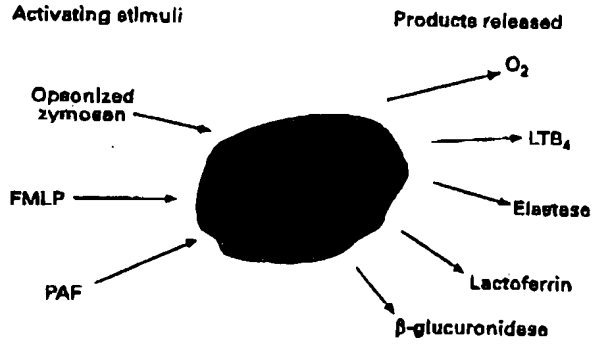

Fig. 2. The neutrophil as a potential target in the treatment of COPD with selective PDE inhibitors. PDE4 is the predominant cAMP-metabolizing enzyme in human neutrophils and is involved in several functions and responses to exogenous stimuli. FMLP, N-formyl-methionyl-leucyl-phenylalanine; PAF, platelet-activating factor; LTB_4, leukotriene B_4.

combined inhibition of PDE3 and 4, or 3 and 5 exerted relaxation of pulmonary artery rings, whereby these functional results corresponded broadly to the pattern of PDE isozymes found in the pulmonary artery smooth muscle [49].

Lymphocytes

Homogenates of lymphocytes from the peripheral blood predominantly show PDE3 and PDE4 activities with no difference between CD4 and CD8 positive cells. In addition to its ability to inhibit mitogen-induced and T-cell antigen receptor (CD3)-mediated proliferation of T lymphocytes *in vitro* [50,51], theophylline enhances the suppression of autologous cell proliferation in mixed lymphocyte preparations [52], a phenomenon that is reflected by the induction in theophylline-treated asthmatic subjects of an increased population of suppressor T cells [53,54]. Theophylline also reduces cytokine release from T lymphocytes *in vitro* [51,55], which may imply further actions on cytokine-dependent functions of other leucocytes *in vivo*.

Mast cells

Experiments on human lung mast cells suggest that these cells contain PDE3 and PDE4 [25,56], since both PDE3 and PDE4 inhibitors reduce antigen-driven histamine release from human lung [57,58] and skin [58] mast cells.

Neutrophils

In human neutrophils PDE4 is the predominant cAMP-metabolizing enzyme [59–61], which is involved in many neutrophil functions and neutrophil responses to stimuli (Figure 2). These processes include the stimulation of superoxide anion production by N-formyl-methionyl-leucyl-phenylalanine (fMLP) [59–62], C5a [60], granulocyte-macrophage colony-stimulating factor [63], and TNFα [64]. Furthermore, PDE4 plays an important role in the regulation of fMLP-induced degranulation [65–67], leukotriene biosynthesis [61], and adhesion to endothelial cells [68]. Inhibitory effects can be observed *ex vivo* on neutrophils from patients treated with theophylline, whose chemotactic responses are reduced compared with cells from untreated patients [69].

Eosinophils

Human eosinophils contain PDE4 and a broad spectrum of eosinophil functions (Figure 3) are suppressed by PDE4

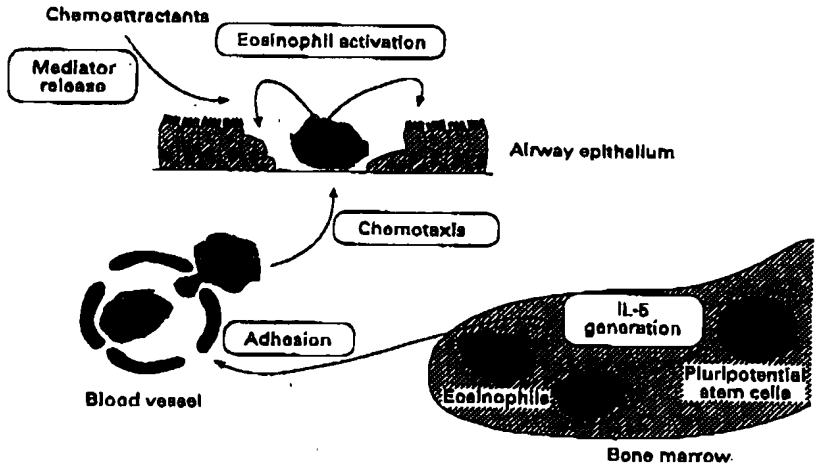

Fig. 3. The eosinophil as a potential target in the treatment of bronchial asthma with selective PDE inhibitors [12]. PDE4 is the predominant cAMP-metabolizing enzyme in human eosinophils. PDE4 inhibitors affect proliferation, differentiation, and export of eosinophils from the bone marrow, chemotaxis, adhesion, and transmigration in the blood vessels, and the release of chemotactic and inflammatory mediators from activated cells.

inhibitors such as opsonized zymosan-stimulated superoxide generation [70,71], platelet-activating factor (PAF)- and C5a-induced LTC_4 production, as well as chemotaxis [25,72]. Furthermore, in the presence of a β-adrenoceptor agonist, PDE4 inhibitors reduced the release of eosinophil cationic protein and eosinophil-derived neurotoxin in response to C5a [73].

High concentrations of theophylline suppress reactive oxygen metabolite production by neutrophils and eosinophils but lower concentrations enhance this response [74,75]. At high concentrations, the survival time of eosinophils in culture is reduced [76], apparently through the induction of apoptosis, and this may have implications for the maintenance of inflammation *in vivo*. Other eosinophil functions, such as leukotriene C_4 release, however, are inhibited by low concentrations of theophylline *in vitro* [77].

Side effects of PDE inhibitors

Theophylline exerts frequent side effects that can become severe at plasma drug concentrations only slightly higher than those typically required to effect bronchodilation in asthmatic patients. Side effects of theophylline stem from its tendency to inhibit indiscriminately all PDEs in all tissues of the body [78,79]. The side effects produced by nonselective inhibition limits the degree of enzyme inhibition achievable in target tissues, i.e. airway and vascular smooth muscle as well as immunocompetent cells. The most common side effects, including cardiac dysrhythmias and nausea, are believed to be mediated by PDE inhibition.

Chronotropic effects on the heart, particularly sinus tachycardia [80], can be caused by the elevation of cAMP levels in cardiac muscle with a subsequent calcium overload [81] or reflecting a reflex reaction to peripheral vasodilation [82]. Theophylline has long been recognized as a diuretic, and an action that results at least in part from increased renal blood flow and glomerular filtration rate. *In vivo*, however, theophylline treatment can cause electrolytic disturbances, with hypokalaemia being a particular problem [83]. Furthermore, theophylline as well as caffeine, stimulates the central nervous system at relatively low doses *in vivo*, since the drugs are concentrated in the cerebrospinal fluid [80]. Actions of methylxanthines in the central nervous system include a beneficial increase in ventilatory drive and a general increase in alertness and lessening of fatigue, but also account for the risk of tremor and seizures during theophylline therapy. Effects on the central nervous system are further nausea and vomiting, whereby this action can be mimicked by selective PDE4 inhibitors and apparently results from the inhibition of PDE in the emesis centres of the brain [84]. Another side effect is the increased acid secretion that is believed to be due to inhibition of PDE4 in parietal glands [85].

PDE3 inhibition can produce a number of cardiovascular side effects [86,87] that include life-threatening arrhythmias in patients with compromised cardiac function [88]. The cardiovascular activity of dual PDE3/4 inhibitors is likely to be even more marked, since PDE4 inhibitors, although devoid of cardiovascular effects on their own, potentiate the cardiovascular actions of PDE3 inhibitors [89]. Nausea, vomiting, and gastric acid secretion are class effects of first-generation PDE4 inhibitors [17–19,85,90,91].

Rationale for PDE4 inhibitors in asthma and COPD

Airflow obstruction and airway inflammation are features of asthma as well as COPD. While bronchial asthma is predominantly characterized by an eosinophilic inflammation, neutrophils are believed to play a major role in the pathogenesis of COPD. Therefore, PDE isozymes that are involved in smooth muscle relaxation and are also found in eosinophils as well as neutrophils, are likely to play an important role in both diseases and represent an obvious target for new therapeutic strategies. The development of selective PDE-inhibiting substances might have additional clinical relevance, minimizing side effects that are seen in patients treated with nonselective inhibitors such as theophylline.

It has been suggested that many of theophylline's adverse effects, including cardiac dysrhythmias and nausea, are due to the drug's nonselective PDE inhibition. In addition to theophylline's relatively low efficacy, it was proposed that selective targetting of the PDE isoenzymes involved in regulating cyclic nucleotide levels in cells relevant to asthma and also COPD – namely bronchial smooth muscle and the inflammatory cells characteristic of bronchial infiltrates – would allow the beneficial effects of theophylline to be obtained in the absence of cardiovascular, gastrointestinal, and central nervous system side effects.

The ability of PDE4 inhibitors both to relax airway smooth muscle on one hand and to suppress the function of a range of inflammatory cells on the other, led to a concentration of the research effort on drugs of this class. Selective PDE4 inhibitors have been demonstrated to exert suppressive action on neutrophil and eosinophil function *in vitro* that resembles the action of high concentrations of theophylline [61,70,77], with the added benefit of exhibiting none of the actions of theophylline that are assumed to result from that drug's adenosine antagonistic action [74,75]. *In vivo*, PDE4 inhibitors reduce the influx of eosinophils to the lungs of allergen-challenged animals in a manner similar to that displayed by theophylline, whilst also reducing the bronchoconstriction and elevated bronchial responsiveness occurring after allergen challenge [92–94]. These effects can be seen in the absence of cardiovascular effects such as tachycardia, although emetic and other unwanted gastro-

intestinal effects persist with some of these drugs [91,95]. PDE4 inhibitors also suppress the activity of immune cells, including $CD4^+$ T-lymphocytes, monocytes, mast cells, and basophils. These agents also reduce pulmonary oedema in animal models, inhibit eNANC activity, potentiate iNANC activity, have been reported to reduce airway smooth muscle mitogenesis, and induce bronchodilation.

PDE4 inhibitors also suppress the activity of a number of inflammatory cells associated with the pathophysiology of COPD, including monocytes/macrophages, $CD8^+$ T lymphocytes, and neutrophils. PDE4 inhibitors also reduce vascular smooth muscle mitogenesis and, potentially, alter the ability of airway epithelial cells to generate pro-inflammatory mediators. Through the release of neutral proteases and acid hydrolases from their granules and the generation of reactive oxygen species, neutrophils contribute to tissue destruction associated with chronic inflammation and are implicated in the pathology of conditions such as emphysema [96].

Clinical trials in asthma

In summarizing the available clinical data on selective PDE inhibitors for asthma and COPD, it becomes apparent that there is a significant imbalance between the increased knowledge of the molecular biology of PDE, including the performed *in vitro* experiments, and larger clinical trials. While there are a few data to be reported in the field of asthma, the experience in COPD is even more scarce and larger clinical trials are just about to begin.

Earlier studies with selective PDE3 inhibitors such as milrinone, have demonstrated that these drugs induce short-lived bronchodilation and some minor degree of protection against induced bronchoconstriction [97,98]. These compounds, which were developed as cardiotonic agents on the basis of their profound effects in the cardiovascular system [99], produced marked side effects, such as tachycardia, hypotension, and headache [97,98], and appeared unlikely to be of benefit in the therapy of obstructive lung diseases.

A preliminary and early report of the actions of a weak selective PDE4 inhibitor, tibenelast (LY 186,655), described a slight and statistically nonsignificant increase in baseline forced expiratory volume in 1 s (FEV_1) in asthmatic subjects [100], while clinical trials which were performed with the PDE5 selective inhibitor zaprinast showed that oral administration of the compound reduced exercise-induced bronchoconstriction in adult asthmatics. Surprisingly, in the some studies the drug had no effect on histamine-induced bronchoconstriction in the same subjects [101], nor did it affect exercise-induced bronchoconstriction in children [102].

Further clinical data were obtained with dual PDE3/4 selective inhibitors, but the results obtained with drugs such as zardaverine and benafentrine were inconclusive. Inhalation, but neither oral nor intravenous application, of benafentrine by normal volunteers resulted in bronchodilation [103]. Zardaverine produced a modest and short-lived bronchodilation in a small group of 12 asthmatic patients, which was significant only during the first hour after administration (Figure 4a). The drug was given by inhalation and patients inhaled 1.5 mg of zardaverine four times at 15-min intervals [104].

Recently, the efficacy and safety of a novel selective PDE4 inhibitor, SB 207,499 (Ariflo™), in asthma was

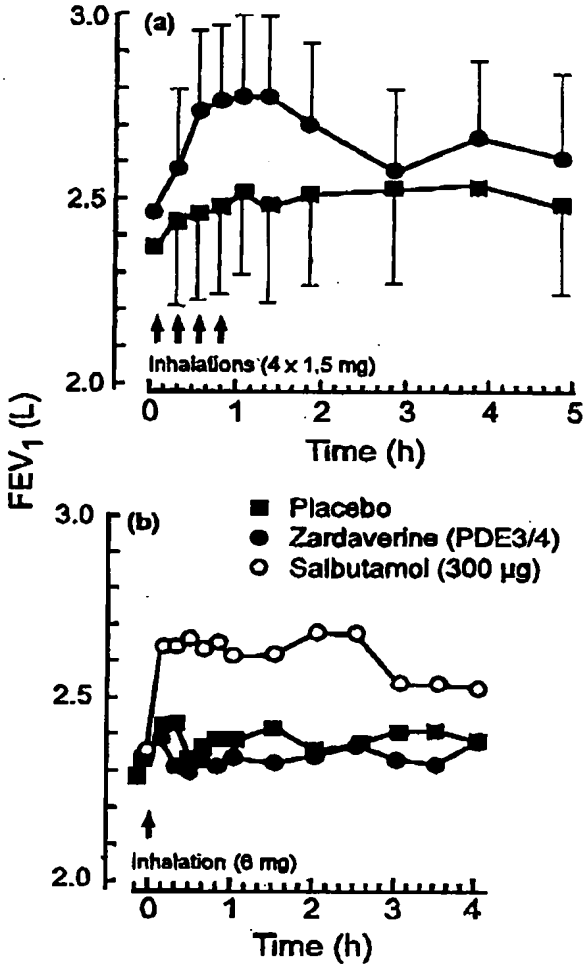

Fig. 4. Effect of the dual PDE3/4 inhibitor zardaverine on baseline lung function in patients with (a) bronchial asthma [104] and (b) COPD [107]. Compared with placebo, zardaverine given by inhalation produced modest bronchodilation in asthmatic patients (a), whereas no effect on lung function was observed in patients with COPD (b). FEV_1, forced expiratory volume in 1 s.

explored in a 6-week placebo-controlled dose-ranging study in patients taking concomitant inhaled corticosteroids. Three hundred and three patients were treated with oral SB 207,499 at dosages of 5, 10, or 15 mg b.i.d., or placebo, for 6 weeks. In patients receiving 15 mg b.i.d., there was a 160-mL mean difference in trough FEV_1 compared with placebo at the end of the study, which did not quite reach statistical significance. The increase in trough FEV_1 was evident at week 1 and was significantly different from placebo at week 2 (mean difference 210 mL; $P = 0.006$). Trends for improvements were also seen for other pulmonary function tests, including PEFR and FEF_{25-75}. Interestingly, the effects of SB 207,499 on FEV_1 and PEFR were greater in patients taking less inhaled steroids.

A double-blind, placebo-controlled study has been made of another potent, selective PDE4 inhibitor, CDP840, which showed a suppression of late reactions to inhaled allergen after 9.5 days' oral administration. The late reaction, measured as area under the curve, was inhibited by 30% in a group of 14 asthmatics with no effect on the early response to allergen (Figure 5). Interestingly, two other studies performed in parallel demonstrated that this drug given orally at doses of 15 and 30 mg had no effect on baseline lung function or histamine-induced bronchoconstriction in patients with asthma [105].

Clinical data in COPD

Information from clinical trials with isoenzyme-selective PDE inhibitors in patients with COPD is still very limited. At this time only very few published data exist on the treatment of COPD with selective PDE inhibitors, while several larger studies are being performed at present.

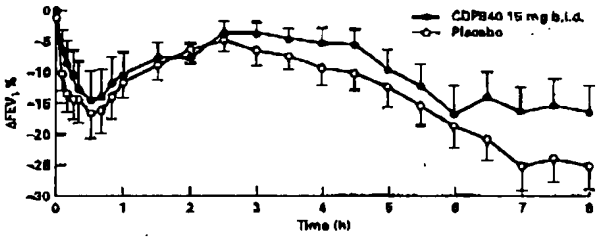

Fig. 5. Effect of the selective phosphodiesterase 4 inhibitor CDP840 on allergen responses in patients with bronchial asthma [105]. In asthmatic patients the late-phase response of allergen-induced bronchoconstriction was suppressed after 9.5 days of treatment with CDP840 (closed circles) as compared with placebo (open circles). No difference was found between CDP840 and placebo regarding baseline lung function or the early phase response to allergen. Responses to allergen were expressed as the percentage of fall in FEV_1 over the 8-h period following exposure. Values are given as mean ± SEM. ΔFEV_1, change in forced expiratory volume in 1 s; %, per cent of baseline FEV_1.

Pulmonary hypertension is observed in patients with severe COPD and oral administration of the selective PDE3 inhibitor, milrinone, decreases mean pulmonary artery pressure under clinical conditions [106]. Furthermore, the selective PDE3 inhibitor, enoximone (MDL 17043), was shown to reduce pulmonary hypertension and airway resistance in patients hospitalized with decompensated COPD [97]. The fact that selective PDE3 inhibitors are able to exhibit these effects is in accordance with *in vitro* findings demonstrating that selective inhibition of PDE3 and 5, or combined inhibition or PDE 3 and 4 or 3 and 5, exerted relaxation of pulmonary artery rings, corresponding broadly to the pattern of PDE isozymes found in the pulmonary artery smooth muscle [49]. Selective PDE3 inhibition by motapizone, and PDE5 inhibition by zaprinast, decreased pulmonary artery pressure to the same extent, whereas PDE4 inhibition was less effective. Although these *in vitro* data suggested a marked effect of PDE3/4 and most notably PDE3/5, no studies with these combinations have so far been reported.

In a randomized, double blind and placebo-controlled phase II clinical trial, 10 patients with chronic airflow obstruction were investigated to assess the effect of the dual PDE3/4 inhibitor zardaverine [107]. The study medication was given by MDI at three different doses and the effect on FEV_1 measured by spirometry was assessed at 10-min intervals for the first hour, and at 30-min intervals for 3 h after medication. In contrast to the findings obtained in patients with bronchial asthma, the inhalation of zardaverine had no bronchodilatory effect in these patients with COPD, although it was demonstrated in the same study that the subjects responded to inhalation of 300 μg salbutamol with a significant increase in forced expiratory volume (Figure 4b).

The selective second-generation PDE4 inhibitor, Ariflo™ (SB 207,499), is currently under investigation also as a potential therapy for obstructive lung disease. The first study performed by Murdoch *et al.* [108] was a randomized double-blind, placebo-controlled, parallel group study to assess the safety and tolerability of single and repeat oral doses of SB 207,499. At doses up to and including 15 mg b.i.d. this PDE4 inhibitor was safe and well tolerated; however, at a dose of 20 mg given as a single dose, relevant side effects such as nausea and vomiting occurred.

The efficacy and safety of SB 207,499 in COPD was then evaluated in a randomized, placebo-controlled study [109]. Four hundred and twenty-four patients with COPD were randomized to receive Ariflo™ at dosages of 5, 10, or 15 mg b.i.d., or placebo, for 6 weeks. Patients in this trial had moderate to severe airflow obstruction with an FEV_1 at baseline of 46.8% predicted normal, and a FEV_1:FVC ratio of 55%. Notably, they were only partially reversible with a mean acute responsiveness to salbutamol of 5.4%, and had a

mean smoking history of 39.7 pack years. In the group of patients receiving Ariflo™ 15 mg b.i.d., a progressive improvement in trough clinic FEV_1 was observed, beginning in 1 week and reaching a maximum mean difference compared with placebo of 160 mL ($P<0.001$) at week 6, representing an 11% improvement. Similar trends were observed for trough FVC and PEFR, with maximum mean differences compared with placebo observed at week 6 of 190 mL ($P<0.001$) and 34.1 L/min ($P<0.001$), respectively. In addition, consistent improvements relative to placebo were observed for the 15 mg b.i.d. dose in exertional dyspnoea, rescue bronchodilator use, and resting and postexercise SaO_2.

Conclusions

The importance of PDE in the regulation of cell function is increasingly recognized, and in the past this has led to several programmes of synthesis of novel PDE inhibitors – of varying isoenzyme selectivity – as potential anti-asthma drugs. Data from clinical trials with the first selective drugs have been disappointing and have tempered the expectations considerably, since these drugs had limited efficacy and their use was significantly limited through side effects. The improved understanding of the molecular biology of PDEs together with a continued effort to specifically target novel drugs to improve the risk/benefit ratio has now resulted in second-generation drugs that have produced more promising clinical results that seem to justify the wide research efforts in the past. Probably even more importantly, selective inhibitors of PDE4 are now also under clinical investigation for COPD and the results so far are promising that this class of drug may add to the therapy of chronic airflow obstruction. If confirmed in larger and more detailed trials this may turn out to be of particular importance, since the existing drug therapy, including steroids, has so far failed to alter significantly the course of the disease in chronic treatment.

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